

STIC-ILL

Adonis
mc

From: Baskar, Padmavathi
Sent: Monday, June 23, 2003 12:50 PM
To: STIC-ILL
Subject: 09/300612,

Rodriguez-Acosta A, Aguilar I, Giron ME.

Antivenom activity of opossum (*Didelphys marsupialis*) serum fractions against Uracoan rattlesnake
(*Crotalus* vegrandis Klauber, 1941) venom.
Roum Arch Microbiol Immunol. 1995 Oct-Dec;54(4):325-30.

Toxicon. 1996 Sep;34(9):1067-71.

Halpern M, Shapiro LS, Jia C.

Differential localization of G proteins in the opossum vomeronasal system.
Brain Res. 1995 Apr 17;677(1):157-61.

Antigenic relationship among antihemorrhagic factors from snake and opossum plasmas.
Braz J Med Biol Res. 1989;22(6):717-9.

Padma Baskar
Art Unit 1645
Patent Examiner/Biotechnology
CM-1, 8E-13
703-308-8886

Search

Leu Lys Ala Met Asp Pro Thr Pro Arg Leu
L K A M D P T P P L

Trp ~~Leu~~ Lys Thr Glu
W I ~~TE~~

L K A M D P T P P L W I ~~TE~~

2

3



ISOLATION OF PROTEIN FACTORS FROM OPOSSUM (*DIDELPHIS ALBIVENTRIS*) SERUM WHICH PROTECT AGAINST *BOTHROPS JARARACA* VENOM

MARIA DE FÁTIMA LOVO FARAH,¹ MASSAYUKI ONE,¹
JOSÉ CAMILLO NOVELLO,¹ MARCOS HIKARI TOYAMA,¹
JONAS PERALES,² HAITI MOUSSATCHÉ,³ GILBERTO BARBOSA
DOMONT,² BENEDITO OLIVEIRA¹ and SERGIO MARANGONI^{1*}

¹Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, 13087-970, Campinas, SP, Brazil. ²Departamento de Bioquímica, Instituto de Química, UFRJ, 21910, Rio de Janeiro, RJ, Brazil and ³Departamento de Fisiologia e Farmacodinâmica, Fundação Oswaldo Cruz, Av. Brasil, 4365, 21045-970, Rio de Janeiro, RJ, Brazil

(Received 4 December 1995; accepted 19 February 1996)

M. F. L. Farah, M. One, J. C. Novello, M. H. Toyama, J. Perales, H. Moussatché, G. B. Domont, B. Oliveira and S. Marangoni. Isolation of protein factors from opossum (*Didelphis albiventris*) serum which protect against *Bothrops jararaca* venom. *Toxicon* **34**, 1067-1071, 1996.—The fractionation of *Didelphis albiventris* serum by DEAE-Sephadex A50 yields a fraction (DA2) which protects the opossum against *Bothrops* venom. One polypeptide (DA2-II) responsible for this protection was isolated from fraction DA2 by ion exchange chromatography and biochemically characterized. DA2-II is a 43,000 mol. wt glycoprotein with the following N-terminal sequence: LKAMDTTPPLKIKKEPVK. Pairwise comparison of the amino acid sequence with four anti-hemorrhagic factors isolated from other opossum species indicated that DA2-II possesses high similarity (60-80%) with these proteins. Copyright © 1996 Elsevier Science Ltd

Resistance to the effects of snake venoms has been studied extensively in the North American opossum *Didelphis virginiana* (Werner and Vick, 1977; Huang and Perez, 1980). A proteinase inhibitor (oprin) isolated from *D. virginiana* serum has been shown to protect this marsupial against the deleterious actions of metalloproteinases and hemorrhagic toxins found in *C. atrox* venom (Catanese and Kress, 1992). The present report describes the isolation and partial characterization of a protein factor from opossum (*D. albiventris*) serum which protects against the hemorrhagic and lethal effects of *Bothrops jararaca* venom.

D. albiventris specimens were caught in region of Campinas (south-eastern São Paulo state) and were bled by cardiac puncture. The blood was allowed to clot at room

*Author to whom correspondence should be addressed.

temperature after which the serum was separated by centrifugation at 900 *g* in a Sorval RC-2B centrifuge (20 min 4°C) and stored at -70 °C. The experiments were performed with a pool of sera from these animals. *Bothrops jararaca* venom was obtained from the Instituto Butantan (São Paulo, Brazil)

D. albiventris serum was fractionated by ion exchange chromatography on DEAE-Sephadex A-50 column equilibrated with 0.01 M sodium acetate pH 3.7. Two fractions, DA1 and DA2 (the latter being the active peak), were obtained using a step-wise gradient. DA2 was further purified by HPLC ion exchange chromatography on a Waters SP 5PW column (0.39 × 30 cm) with a linear gradient of 0-0.1 M sodium chloride in 100 mM sodium acetate buffer pH 4.1, at a flow rate of 1.0 mliter/min for 60 min. The elution profile was monitored by the absorbance at 280 nm and the protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

D. albiventris serum and its fractions were loaded onto 10% polyacrylamide slab gel in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970). The periodic acid-Schiff procedure was used to detect the presence of glycoproteins as follows. The gel was fixed in 7.5% acetic acid followed by oxidation with 0.2% sodium metaperiodate for 45 min at 4°C, and then stained with Schiff's reagent for 1 hr at 4°C. Destaining was carried out at room temperature using several changes of 10% acetic acid.

The anti-lethal activity of *D. albiventris* serum and its fractions was determined by injecting five groups of male Swiss white mice (20 g, six mice/group) i.p. with 2 LD₅₀ (6 ± 0.2 µg/g) (Weil, 1952) of *B. jararaca* venom dissolved in 0.9% (w/w) saline solution. Each sample tested contained 5-200 µg protein/g. The serum fraction and venom mixture was incubated at 37°C for 30 min. Venom and saline were injected as positive and negative controls, respectively. The number of surviving mice was scored 24 hr later and the LD₅₀ and 95% confidence intervals were calculated according to the method of Finney (1952). The anti-hemorrhagic activity of whole serum and of the purified fractions was determined by intradermal injection (Ownby *et al.*, 1984). Initially, 0.5-5 µg of venom in 0.1 ml of saline was injected intradermally into the back of male Swiss white mice. One hour later the skin was removed and the size of the hemorrhagic spot was measured [1 cm of diameter was defined as minimal hemorrhagic dose; Kondo *et al.* (1960)]. Subsequently, the anti-hemorrhagic activity was assessed by incubating *D. albiventris* serum or its fractions with 2 mg (minimum dose) of crude venom for 1 hr at 37 °C and then testing as described above.

The N-terminal sequence of DA2-II was determined by automated Edman degradation using an Applied Biosystems model 477 sequencer. Phenylthiohydantoin amino acids were identified with a model 120-A PTH-amino acid analyzer (Applied Biosystems), based on the retention times of PTH-amino acid standards.

The Brazilian opossum *D. albiventris* is naturally resistant to the lethal effects of up to 100 LD₅₀ (inice) of *Bothrops jararaca* venom administered i.p. and is able to recover from envenomation symptoms such as hemorrhage, tissue necrosis, and coagulation defects. Thus, this species may be included with other mammals known to be resistant to snake venoms.

A protective fraction consisting of proteins with a molecular mass of 43,000-67,000 mol. wt able to inhibit thrombin-induced human washed platelet aggregation was obtained by gel filtration of *D. albiventris* serum (Condino-Neto *et al.*, 1992). More recently, Perales *et al.* (1994) reported the isolation of an anti-bothropic venom complex from the serum of South American Didelphidea.

Purification of the protective fractions from *D. albiventris* serum was carried out on a DEAE-Sephadex A-50 column. Fraction DA1 eluted with the equilibrating buffer while fraction DA2 eluted with the same buffer in the presence of 0.15 M NaCl. Polyacrylamide gel electrophoresis of fraction DA2 shows the presence of only two protein bands estimated molecular mass of 40,000–50,000 mol. wt in native and denaturing conditions and is thus similar to the anti-bothropic venom complex isolated from *D. marsupialis* serum (Perales *et al.*, 1994). Fraction DA2 fraction stained with PAS indicating that these anti-hemorrhagins must be glycoproteins. Reduction with DTT showed that they are not composed of subunits linked by disulfide bridges. Weissenberg *et al.* (1991) obtained anti-hemorrhagins from *Crotalus atrox* serum which consisted of homologous glycoproteins that differed in their carbohydrate content.

D. albiventris serum protects against the lethal effects of 1 LD₅₀ of *B. jararaca* venom. Only fraction DA2 (dose, 10 µg/g) obtained after DEAE-Sephadex A-50 chromatography was able to neutralize the lethality. This efficacy is similar to that of other anti-hemorrhagic factors (Ovadia, 1978; Garcia and Perez, 1984; Tanizaki *et al.*, 1991; Catanese and Kress, 1992).

SDS-PAGE electrophoresis of the subfractions obtained after the chromatography of fraction DA2 on HPLC SP 5PW ion exchange column, revealed single bands with mobilities compatible with molecular masses of 48,000 and 43,000 mol. wt, which were termed DA2-I and DA2-II, respectively (Fig. 1). DA2-I had no protective activity in mice (venom dose, 10 µg/g) whereas DA2-II fully protected against the hemorrhagic activity of this same dose of *B. jararaca* venom. Our work thus corroborates a previous study by

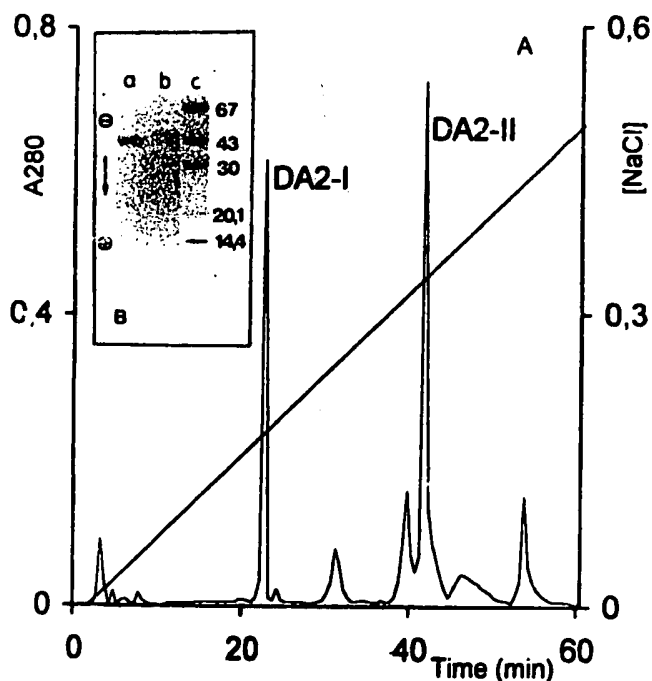


Fig. 1. Ion exchange chromatography and SDS-PAGE analysis of the anti-hemorrhagic factor isolated from *D. albiventris* serum. (A) Ion exchange chromatography of fraction DA2 on a Waters SP 5PW column (0.75 × 7 cm) yielded two protein fractions (DA2-I, DA2-II). Elution was initiated with 100 mM sodium acetate buffer, pH 4.1, followed by a linear gradient (0–1.0 M) of sodium chloride. Fractions (0.5 mliter) were collected at a flow rate of 1.0 mliter/min. (B) SDS-PAGE (10%) of DA2-II (lane a), DA2-I (lane b), and molecular mass standards (lane c).

Fig. 2. Comparison of the N-terminal amino acid sequence of fraction DA2-II from *D. albiventris* serum with other mammalian anti-hemorrhagic proteins. Alignment and numbering refer only to DA2-II. Invariable residues in all anti-hemorrhagic proteins are shaded. × represents an unknown residue. Amino acid sequence for the proteins of *D. marsupialis*, *L. crassicaudata*, and *P. opossum* were determined by Perales *et al.* (1994). The amino acid sequence for oprin (from *D. virginiana*) was determined by Catanese and Kress (1992).

The above results show that the anti-hemorrhagic factors from different species of Didelphidae (North and South American) are related. The invariable native of the first five aminoacids suggesting that this region may represent a conserved three-dimensional structure responsible for the biological activity located in the core region of the molecule (Perales *et al.*, 1994).

REFERENCES

- Catanese, J. J. and Kress, L. F. (1992) Isolation from opossum serum of a metalloproteinase inhibitor homologous to human α 1 B-Glycoprotein. *Biochemistry* **31**, 410-418.
- Condino-Neu, A., Landucci, E. C. T., Farah, M. F. L., Annichio-Bizzachi, J., Perez, A. C., Hyslop, S., Marangoni, S., Oliveira, B., Domont, G., Perales, J. H., Moussatché, H. and Nucci, G. (1992) The inhibition of thrombin-induced human platelet aggregation by proteins present in serum from the opossum *Didelphis albiventris*. *Thromb. Haemorrh. Dis.* **6**, 41-46.
- Finney, D. I. (1952) *Statistical Methods in Biological Assay*. London: Charles Griffin.
- Garcia, V. E. and Perez, J. C. (1984) The purification and characterization of an antihemorrhagic factor in woodrat (*Neotoma micropus*) serum. *Toxicon* **22**, 129-138.
- Huang, S. and Perez, J. C. (1980) Comparative study on hemorrhagic and proteolytic activities of snake venoms. *Toxicon* **18**, 421-426.
- Kondo, H., Kondo, S., Ikezawa, H., Murata, R. and Ohsaka, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of *Habu* snake venom. *Jap. J. med. Sci. Biol.* **13**, 43-47.

- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature* **227**, 608-609.
- Lowry, O. H., Rosebrough, N. J., Farr, L. A. and Randall, R. S. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265-275.
- Ovadia, M. (1978) Purification and characterization of an antihemorrhagic factor from the serum of the snake *Vipera palaestinae*. *Toxicon* **16**, 661-672.
- Owby, C. L., Colberg, T. R. and Odell, G. V. (1984) A new method for quantification of hemorrhage induced by rattlesnake venoms: ability of polyvalent antivenoms. *Toxicon* **22**, 227-233.
- Perales, J., Moussatché, H., Marangoni, S., Oliveira, B. and Domont, G. B. (1994) Isolation and partial characterization of an anti-bothropic complex from the serum of South American Didelphidae. *Toxicon* **32**, 1237-1249.
- Tanizaki, K. M., Kawasaki, H., Suzuki, K. and Mandelbaum, F. R. (1991) Purification of a proteinase inhibitor from the plasma of *Bothrops jararaca* (jararaca). *Toxicon* **29**, 673-681.
- Weil, C. S. (1952) Table for convenient calculation of median effective doses (LD_{50} and ED_{50}) and instruction for their use. *Biometrics* **8**, 249-263.
- Weissenberg, S., Ovadia, M., Fleminger, G. and Kochva, E. (1991) Antihemorrhagic factors from the blood serum of the western diamondback rattlesnake *Crotalus atrox*. *Toxicon* **29**, 807-818.
- Werner, R. M. and Vick, J. A. (1977) Resistance of the opossum (*Didelphis virginiana*) to envenomation by snakes of the family Crotalidae. *Toxicon* **15**, 29-33.